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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:	WO 93/12142
C07K 13/00, A61K 45/05	A1	(43) International Publication Date:	24 June 1993 (24.06.93)

(21) International Application Number: PCT/US92/10889

(22) International Filing Date: 10 December 1992 (10.12.92)

(30) Priority data:
805,452 10 December 1991 (10.12.91) US

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(81) Designated States: AU, BB, BG, BR, CA, DK, ES, FI, HU, JP, KP, KR, LK, MG, MW, NO, RO, SD, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: CYTOKINES WITH AN UNPAIRED CYSTEINE RESIDUE AND CONJUGATES THEREOF

(57) Abstract

Disclosed are cytokines which are site-specifically mutated to have one unpaired cysteine residue located apart from the receptor-binding site, and conjugates thereof, in particular, conjugates where a lipophilic group is conjugated to the unpaired cysteine residue, and to such conjugates of cytokines which have an unpaired cysteine residue located apart from the receptor-binding site in the native form. All of these cytokines are of the type such that conjugation of a lipophilic group to the unpaired cysteine residue does not significantly affect the receptor binding or biological acitivity of the conjugated product. Also disclosed are conjugates where a lipophilic group, an antibody, horseradish peroxidase, alkaline phosphatase, fluorescein substances, biotin, or a cytotoxin is conjugated to the unpaired cysteine residue. These fatty acyl conjugated cytokines penetrate better and are retained longer in local sites and can be locally administered to treat, e.g., solid tumors, spinal injuries, the external genital area, the throat, ears, nasal linings, eyes, and skin.

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CYTOKINES WITH AN UNPAIRED CYSTEINE RESIDUE AND CONJUGATES THEREOF

Field of the Invention

The invention relates to cytokines which are site-specifically mutated to have one unpaired cysteine residue located apart from the receptor-binding site, and conjugates thereof, in particular, conjugates where a lipophilic group is conjugated to the unpaired cysteine residue, and to such conjugates of cytokines which have an unpaired cysteine residue located apart from the receptor-binding site in the native form.

Background of Invention

Cytokines act on cells to regulate cell growth, maturation or cellular activity, by either stimulating or inhibiting growth or maturation or activity of neighboring, functionally related cell types. Cytokines are synthesized and secreted by lymphocytes, macrophages, fibroblasts, neuronal cells, or other cell types.

The *in vitro* studies on cytokines indicate that they are generally very potent factors which act at very low concentrations. Each cytokine can have many different effects (pleiotropicity), and act locally on several functionally related cell types. For example, at the site of tissue repair, or inflammation, or of immune response, a cytokine is generally involved in a loop of regulatory processes. The cytokine regulates not only the maturation and activity of the target cells, but the production of the cytokine itself is also regulated by certain local factors. For example, in the microenvironment of a site into which an immunogen is administered, the macrophages are activated and induced to secrete interleukin-1 ("IL-1"). IL-1

activates the neighboring antigen-specific T cells. These activated T cells then secrete interleukin-2 ("IL-2") which acts upon the T cells in an autocrine fashion and the activated T cells also secrete γ-interferon ("IFN-γ") which further activates the macrophages and B cell growth factors, which in turn activate the neighboring antigen-specific B cells.

The localized function of cytokines is to be contrasted with hormones of the endocrine system. A hormone is synthesized by a localized tissue or organ and carried by the blood circulation to act on a tissue or organ systemically, or one which is located in a different part of the body.

Cytokines generally are small proteins, and are made in small quantities. They are generally not detectable in the circulation even with very sensitive immunochemical assays. Cytokines have very high affinity for their receptors on the target cells, and need only bind to a small portion of the receptors on the surface of the target cells to trigger the subsequent biological events.

Many cytokines have been identified, and the genes of many cytokines have been cloned and expressed in various systems. Recombinant cytokines have been produced, purified, and used for various *in vitro* and *in vivo* therapeutic purposes. Certain of these recombinant cytokines have been approved by the United States Food and Drug Administration or the regulatory agencies of other countries for various therapeutic purposes. For example, erythropoietin ("EPO") is approved for increasing red blood cell counts in patients receiving kidney dialysis; granulocyte colony stimulation factor ("GCSF") and granulocyte macrophage colony stimulation factor

("GMCSF") are approved for boosting granulocyte counts in cancer patients receiving chemotherapy or irradiation; IL-2 is approved for enhancing immunity in patients with certain types of cancer; α-interferon ("IFN-α") is approved for patients with certain viral infections (such as hepatitis C) or with certain types of cancer.

Cytokines which target cells in the blood circulation or bone marrow, such as EPO and the aforementioned colony stimulation factors (categorized as hemopoietic growth factors), are safer and more efficacious (or at least have been easier to develop) than those which target cells in the lymph nodes or other tissues. Numerous *in vivo* human clinical studies have indicated that many cytokines, such as IL-2, IL-1, tumor necrosis factor ("TNF"), IFN-α, and IFN-γ, are extremely toxic when administered systemically. In addition, due in part to their small size, cytokines have very short pharmacological half lives and are cleared very rapidly from the circulation.

The cytokines which do not target cells in the blood or circulation seem to be toxic when systemically administered because they cannot act on target cells over short distances or locally, or in a microenvironment in tissues, as they would *in vivo*. Systemic administration results in the cytokine diffusing throughout the circulation and elsewhere, rather than localizing it to the target site.

Several cytokines have been or are being investigated for treating diseases affecting local or regional areas. Epidermal growth factor ("EGF"), fibroblast growth factor ("FGF"), insulin-like growth factor ("IGF"), and platelet-derived growth factor ("PDGF") have been studied in clinical trials for accelerating healing of wounds

resulting from accidents or surgery. Some of these cytokines are also being studied for treating stomach ulcer.

It seems that if the delivery of cytokines and therapeutic enzymes could be localized to maintain sufficient concentration for an appropriate period at the target site, they could be more therapeutically effective. Also, because the toxic effects associated with systemic administration of cytokines would be eliminated, high doses of cytokine could be administered to the diseased tissue site. Some potential applications of localized cytokine administration would include treating solid tumors with TNF, IFN-α, IFN-γ, or IL-2. An injured part of the spine could possibly be treated with nerve growth factor ("NGF") or ciliary nerve trophic factor ("CNTF"). Where the infection by microorganisms is localized to affect only some tissues, such as only the external portions of the mouth, nose, or eyes, or only the external genital organs, or only the toes, or only part of the skin, the administration IFN-α, IFN-β, IFN-γ, or IL-2 to these tissues may be effective. After a surgical procedure, the local administration of PDGF, EGF, FGF, or IGF to the areas of the injured tissues may promote healing. The inflammation of the nasal lining caused by an allergic reaction or by infection may also be treated with EGF, FGF, or IGF, in combination with anti-allergy medication or antibiotics. Injured gums caused, for example, by orthodontic surgery may be treated with EGF or IGF. EGF or IGF could also be used to treat eye trauma caused by ophthalmic surgery or injury. Baldness could be treated by subcutaneous or topical administration of EGF or IGF to the affected areas, in order to promote hair growth.

Fatty acylation of cytokines potentially offers a means to localize the cytokine, thereby preventing the toxicity associated with systemic administration. Localization of the cytokine for a sufficient period also keeps it in an area where it can be therapeutically effective. Proteins which are artificially acylated by fatty acids have been shown to have increased affinity for the cellular plasma membrane. For example, ricin A chain (which is a toxin) after modification by fatty acid conjugation, has greatly increased non-specific cytotoxicity on various cell types. Kabanov, A.V. et el. *Biomed. Sci.* 1:33 (1990). Among the cytokines, TNF has been fatty acylated in order to enhance its ability to incorporate into liposomes. Utsumi, T. et al. *Cancer Res.* 51:3362 (1991). The liposomes were to be administered intravenously into patients to deliver the TNF to cells of the reticuloendothelial system. The fatty acyl group was conjugated by coupling the ϵ amino group of the lysine residues, or the α-amino group of the first amino acid residue of the TNF polypeptide chain, with the N-hydroxysuccinimide ester of the fatty acid. Since they are 6 lysine residues per TNF polypeptide chain, the final products varied in terms of number of fatty groups per molecule and the particular lysine residues to which the fatty acids were conjugated.

It is known that some native proteins are posttranslationally modified *in vivo* by the covalent conjugation of fatty acyl groups. The linkages through which fatty acids are attached to proteins can be divided into two general categories. Wilcox, C. A. and Olson, E. N. *Biochemistry* 26:1029 (1987). The first category of acylated proteins contain fatty acid linked to the polypeptide by an O-ester or thiol (SH) ester

bond. These acylated proteins are localized to cell membranes and are acylated primarily by saturated palmitic acid, a 16-carbon residue fatty acid.

The second category of acylated proteins contain fatty acids linked to the polypeptide by an amide bond. These proteins are acylated with myristic acid, a 14-carbon saturated fatty acid, which is linked to an amino terminal glycine residue. In contrast to proteins acylated with palmitate, myristate-containing proteins have been shown to be both soluble and membrane-bound.

Among the cytokines, an acylated native palmityl-IL-1 has been discovered. This isoform of IL-1 is membrane-bound on the surface of monocytes and macrophages. Bakouche, O. et al. *J. Immunol.* 147:2164 (1991). The O-ester and S-ester linkages to the fatty acyl group are carried out enzymatically in the cis-Golgi apparatus and in the transitional elements of the endoplasmic reticulum, where enzymes recognize certain structural features of the proteins which are to be acylated. Only a small fraction of the total IL-1 is acylated, and only one of many OH groups associated with serine residues, or only one of many SH groups associated with cysteine residues, is conjugated with the palmityl group.

The difficulty with fatty acylation by the present chemical synthetic methods as has been performed with ricin A chain and TNF, therefore, lies in ensuring that the cytokines are in fact acylated and form a homogeneous conjugated population. Another difficulty is ensuring that the cytokine binding site does not become fatty acylated, as this could inhibit or prevent its binding and/or its biological activity.

Summary of the Invention

The invention includes cytokines which are site-specifically mutated to contain one unpaired cysteine residue located apart from the receptor-binding site. More specifically, a cytokine molecule is site-specifically mutated by recombinant DNA methods so as to yield an unpaired cysteine residue at a site apart from the receptor binding site. This can be achieved, for example, by substituting an unpaired cysteine residue for another amino acid residue, such as serine, at a site apart from the receptor binding site. The cytokines that contain an unpaired cysteine residue located apart from the receptor-binding sites accessible for conjugation with a property-modifying group, whether the unpaired residue is native or introduced by site-specific mutation, are referred to as AUC cytokines, (AUC denotes "allosteric unpaired cysteine").

The invention further includes site-specific lipophilization of such AUC cytokines. Lipophilization increases the lipophilicity (affinity for lipid), which aids the resulting product in attaching to cell membranes or in localization to a target site, and thereby reduces its clearance rate. The lipophilized cytokine can attach to the cells at the site of administration, which slows the rate of diffusion into the vascular space or into the mucous fluid and hence retains the product at the target site for longer duration. The biological activity and binding properties of the lipophilized cytokine, however, are substantially the same as the native form.

One preferred embodiment of the invention has a cytokine lipophilized by covalently conjugating it to one fatty acyl group. The preferred fatty acyl groups may

range from 6-18 carbon atoms in length.

The invention further includes AUC cytokines conjugated to an antibody or another type of binding molecule, horseradish peroxidase, alkaline phosphatase, fluorescein substances, or biotin. Such conjugates are useful as reagents for cellular and cytokine receptor assays. AUC cytokines, such as IL-2, IL-4, and EGF, may also be conjugated with cytotoxins, such as ricin A chain or pseudomonus exotoxin, for targeting tumor cells expressing high densities of the respective receptors.

The invention further includes pharmaceutical compositions of lipophilized cytokines, or the other cytokine conjugates described above. The pharmaceutical composition may include suitable adjuvants, diluents and solvents.

Detailed Description of the Invention

A. Cytokines Suitable for Lipophilization and Relevant Considerations Therefor

As noted above, a number of native (non-modified) cytokines have been investigated or are being developed for treating localized disease sites. These same cytokines (including EGF, FGF, IGF, PDGF, TNF, IFN-α, IFN-β, IFN-γ, IL-2 IL-1, NGF, and CNTF) can be lipophilized with the techniques of the invention to make them suitable for such treatment. Certain enzymes, such as DNAase, which can be taken by inhalation and is potentially useful in dissolving DNA in alveolar mucus in patients with cystic fibrosis, and superoxide dismutase, which can be administered to the inflamed joints of patients with osteoarthritis, can also be lipophilized with the techniques of the invention to make it suitable for such treatment. All of these cytokines are relatively small proteins, and the human genes expressing them have

been cloned, characterized, and expressed in host cells. Therefore, recombinant cytokines can be readily produced in quantities suitable for site-specific mutation and lipophilization is feasible.

The site-specific mutation of the cytokine, which permits conjugation of the lipophilic substance apart from the binding site, can be accomplished in a number of ways, including by recombinant techniques. The lipophilic substance can be conjugated to the cytokine by a number of techniques, including a chemical reaction between the lipophilic substance and the cytokine. The lipophilic group itself, and the final lipophilized product, should be substantially resistant to enzyme cleavage. The lipophilized product should have substantially the same receptor-binding activity and biological activity as the native form.

The lipophilic substance can be a number of agents, including fatty acid groups, and lipophilic and uncharged peptides. The lipophilic group should be nonimmunogenic, nonantigenic, and not so large as to affect biological activity or receptor binding. Further, as noted above, rather than a lipophilic substance, the cytokine can be conjugated to other chemical modifier groups, including an antibody or another type of binding molecule, horseradish peroxidase, alkaline phosphatase, fluorescein substances, biotin, or a cytotoxin molecule.

A preferred lipophilized product is conjugated to a fatty acyl group. The length of the fatty acyl group determines the lipophilicity of the final product, and the lipophilicity of the cytokine itself determines the equilibrium/distribution of it between the cellular plasma membrane and the extracellular space. One can select from fatty

acids with a wide range of chain lengths (including, 6, 8, 10, 12, 14, 16, and 18 carbons) to select the fatty acyl group which provides optimal pharmacokinetic properties of the final product for the targeted application.

Generally, greater lipophilicity is provided by a longer fatty acyl group. The greater lipophilicity renders a final product which will be distributed in a more limited area, and will be less diffusive, and will remain longer at the site of administration. A cytokine conjugated with a 16 or 18-carbon acyl group will attach well to the cellular plasma membrane and have limited diffusibility. However, greater lipophilicity is not necessarily more desirable, because some solubility and diffusibility are required to achieve maximum receptor binding and biological effect. The fatty acyl groups providing optimal pharmacokinetics for a typical cytokine are 8-14 carbon long, saturated, and unbranched.

B. Cytokines Suitable for Local Administration and Methods Thereof

A cytokine is suitable for localized as opposed to systemic administration, based on the results for the *in vivo* human clinical studies, if it has one or more of the following properties:

(1) the substance is seriously toxic when administered *i.v.* or by a generalized systemic route;

(2) the substance has a rapid clearance rate, *i.e.*, a serum half life of less than 3-4 hours;

(3) If the substance is systemically administered, the therapeutic effect on the targeted disease is marginal;

(4) The affected site which is to be targeted by the substance is appropriately localized such that the delivery of the substance by a local, nonsystemic administration is feasible; *e.g.*, a nonmetastatic solid tumor or an eye infection is appropriately localized, while erythropoiesis in the bone marrow, although anatomically and histologically localized, is not appropriately localized.

Lipophilization of a cytokine should provide a product with a higher affinity for the cellular plasma membrane than the native cytokine, thereby providing a lipophilized product which will remain at the target site longer than the native product. The lipophilized product should stay attached to the cells for periods of time and not rapidly diffuse into the capillaries to be carried away in the blood stream. Similarly, when a lipophilized product is administered to a mucosal surface, it should be able to attach to the cells and avoid being rapidly washed away by the mucous fluids.

C. Diseases Suitable for Treatment with Lipophilized Cytokines and Methods Thereof

A number of diseases and conditions can be treated with local administration of lipophilized cytokines. If a disease condition is appropriately localized and the affected tissue site is accessible for drug delivery, the local administration of a lipophilized cytokine is desirable, as relatively high doses can be administered without the toxicity associated with systemic administration. The mode of administration should be such that it delivers the drug to all of the affected tissue. "High-density" injection, where small volumes of the therapeutic solution are injected into a large number of sites per unit volume of tissue, is the preferred mode of administration. For example, injections of 10 μl of therapeutic solution into 10 sites of 1 ml of solid

tissue would be a typical protocol for administration.

Solid tumors are one example of a condition suitable for treatment with a lipophilized cytokine. One would directly inject lipophilized IFN-α, IFN-γ, TNF, IL-1 or IL-2 into the solid tumor site. This treatment would be especially attractive when the tumor has not gone into metastasis, or has only limited metastasis. This direct injection can also be administered in conjunction with surgery, in order to provide an even more direct access to the tumor site. Such injection near the excision site also ensures the optimal immune response to aid in destroying the residual tumor cells. In a disease condition, in which the affected tissue is superficial and very thin or exposed, such as an infected cornea, nasal lining, superficial wounds, or bald skin, topical application of a lipophilized cytokine may be effective.

Lesions or genital warts can be treated with direct injection of lipophilized IFN-α, IFN-β, or IFN-γ. Again, there is direct access to the affected site with direct injection. Spinal injuries can be treated with direct injection of lipophized NGF or CNTF. Lipophilized superoxide dismutase may be administered to the inflamed joints of patients with osteoarthritis.

Infections affecting the lungs, such as an influenza or bacterial, viral, or parasitic pneumonia, can be treated with inhalation of lipophilized IFN-α, IFN-β, or IFN-γ. In cystic fibrosis, lipophilized DNase may be taken inhalation. DNase can dissolve DNA in the alveolar mucus, thus reducing the viscosity of the fluid. A preferred inhalation device is a metered dose inhaler, which ensures administration of a measured dosage.

Localized viral, bacterial or yeast infections of the vagina, rectum, mouth, throat, nasal linings, eyes, and/or ears can be treated with application of lipophilized IFN-α, IFN-β, or IFN-γ to the mucosal surface, preferably with a dropper or spraying device. Such topical application of lipophilized EGF, FGF, or IGF can also be effective in promoting healing of wounds or surgical incisions or lesions.

To treat baldness, or to renew or promote hair growth, lipophilized EGF, FGF or IGF can be applied by intradermal or subcutaneous injection, or by topical application.

D. Preparing and Conjugating AUC Cytokines

As noted above, several methods can be employed to conjugate a fatty acyl group to a cytokine. Fatty acyl groups which are modified to contain active linking groups include N-hydroxysuccinimide esters of fatty acid, and can be prepared according to the method of Lapidot, Y. et al. *J. Lipid Res.* 8:142 (1976). Some of these active esters are also available from commercial sources, such as Sigma Chemical Co. (St. Louis, MO) and Matreya, Inc. (Pleasant Gap, PA).

The coupling of the N-hydroxysuccinimide ester to the fatty acid to the cytokine can be performed according to the techniques described in Utsumi, T. et al. *Cancer Res.* 51:3362 (1991). This paper also describes how to titrate to determine the number of fatty acyl groups per molecule on the modified protein. As noted above, it is preferred that only one fatty acyl group be conjugated to each cytokine molecule. The preferred stoichiometry can be achieved by controlling the ratio of reactants during the coupling reaction. The lipohilized cytokines which have one fatty

acyl group per molecule can be purified by a routine procedure.

If the fatty acyl groups are conjugated via the ε-amino groups of lysine residues on the cytokine, one cannot ensure the coupling of one fatty acyl group to the ε-amino group of a particular lysine residue on the cytokine. Therefore, the final lipophilized cytokine will be heterogenic in terms of the number of fatty acyl groups per cytokine molecule and the location of the fatty acyl groups. This type of conjugation creates the possibility that some of the modified cytokine molecules will lose receptor-binding activity, because the fatty acyl groups may couple to amino groups at or near the cytokine receptor binding sites. Further, this type of heterogeneous conjugation increases the possibility of the fatty acyl group-modified sites becoming immunogenic. Therefore, the preferred method of conjugation is to first modify the cytokine in a site-specific manner so that the lipophilic group will bind to the site-specific modification rather than at other locations on the cytokine molecule.

For constructing the lipophilized cytokines of the invention, the cytokine genes are site-specifically mutated by recombinant DNA methods so that the mutated cytokine has an unpaired cysteine residue far enough away from (allosteric to) the receptor binding sites to prevent it from interfering in receptor binding or biological activity.

Generally, native cytokine molecules are single chain polypeptides and contain even numbers of cysteine residues. These cysteine residues form disulfide bonds between pairs of cysteine residues. The specific pairing of the cysteine residues is

determined by the 3-dimensional folding of the polypeptide chain, which is determined by the sequence of the polypeptide. The disulfide bonds are not usually exposed on the surface of the protein molecule, and their function is to hold the protein in a rigid structure to withstand the relatively harsh and variable conditions which exist outside the cytoplasm. Secreted proteins, such as cytokines, have disulfide bonds, whereas proteins which remain in the cytoplasm or on the inner surface of the plasma membrane do not have disulfide bonds.

A cysteine residue can be introduced onto the cytokine at a particular site to provide a docking site for fatty acyl group conjugation. The criteria for choosing where to place this residue are that:

(1) the substitution should not affect the 3-dimensional folding of the cytokine molecule;

(2) the residue should be away from the receptor binding site; and

(3) the residue should be on the surface of the protein molecule and accessible for fatty acylation.

Generally, a serine residue which is in or near a peptide stretch that is highly hydrophilic is most suited for replacement with a cysteine residue. Cysteine and serine residues are structurally highly homologous. The close proximity to or the location in a hydrophilic peptide stretch will ensure that the residue will be on the surface of the protein molecule, so as to be available for chemical conjugation after substitution. Other residues which can be substituted are those which are polar or charged, such as asparagine, glutamine, tyrosine, histidine, lysine, arginine, aspartate,

and glutamate, provided they are in or near a peptide stretch that is hydrophilic.

Since the X-ray crystallographic 3-dimensional structure of most cytokine molecules has not been determined, it is not possible to predict whether an amino acid residue is or is not in or near the receptor-binding site of a particular cytokine. The procedure for making this determination and identifying a suitable residue for substitution involves systemically determining whether the substitution of particular residues by cysteine, followed by lipophilization, affects the receptor binding or the biological activity of the substituted product.

A step-by-step procedure to obtain an AUC cytokine follows.

(i) Sequencing

The first step is to determine the amino acid sequence. For most cytokines, the sequences are available from the literature, and sequencing is not necessary. For others, sequencing can be performed by nucleotide sequencing of the cDNA clones of the mRNA of the cytokines. The deduced amino acid sequences can be confirmed by N-terminal amino acid sequence analysis and from a molecular weight determination of the cytokine proteins.

(ii) Hydrophilicity analysis

The next step is to analyze the hydrophilicity of the cytokine polypeptide. Several software programs that plot the hydrophilicity (or hydropathy) in quantitative indices in relation to the linear amino acid sequence are available and can be used. One of such computer program is developed by Hopp, T.P. and Wood, K.R., and described in *Mol. Immunol.* 20:483 (1983). MicroGenie sequence analysis package

distributed by Beckman Instruments, Inc. Palo, Alto, CA. provides a software program for performing hydrophilicity plots.

(iii) Identifying candidate residues

The next step is to identify the hydrophilic regions in the polypeptide chain and to identify the best-suited residues in or near hydrophilic stretches for the substitution with the cysteine residue. The preferred residue for substitution is a serine residue. However, if a serine residue is not available or not suitable, a histidine, tyrosine, glutamate, aspartate, lysine, histidine, asparagine, or glutamine residue could be an adequate alternative choice. One generates a number of mutant constructs (as many as ten) each having only one substitution per mutant construct. Eventually, using the procedures described further below, the mutant constructs are screened to determine which have a substitution allosteric to the binding site.

(iv) Gene synthesis

The next step is to synthesize the native and mutant genes. Polymerase chain reaction (PCR) can be used to construct the native cytokine gene. One would use oligonucleotide primers that correspond to the 5' and 3' end of the mRNA of the cytokine, and that contain proper cloning sequences. One would start with the RNA preparation from activated lymphocytes, leukocytes, fibroblasts, or other cell lines producing the particular cytokines, from which the cDNA are to be cloned. The cloned cDNA, after sequencing confirmation, is inserted into a plasmid, such as pUC19, for subsequent procedures. One routine laboratory procedure for site-directed mutagenesis is to start with the synthesis of oligonucleotide primers of about 25

nucleotides which contain the triplet condon of a cysteine residue in place of the triplet condon of the serine (or other) residue which is to be replaced. These primers with the installed mutations permit the synthesis of full length DNA genes with the site-directed mutations. A convenient method was developed by Kunkel, T.A., *Proc. Natl. Adac. Sci. U.S.A.*, 82:488 (1985). A step-by-step protocol with the reagents is described by Kunkel, T.A. in *Current Protocols is Molecular Biology*, Supp. 6 § 8.2.1, Eds. Ausubel, F.M. et al, Wiley Intersciences (1990). A PCR method for introducing point mutations in cloned DNA is also routinely used by many molecular biology laboratories. A step-by-step procedure is described by Cormack, B. *Current Protocols in Molecular Biology*, Supp. 15 § 8.5.1 Eds. Ausubel, F.M. et al, Wiley Intersciences (1991).

A preferred method to construct the entire family of native genes and mutant constructs is to synthesize the complete genes with a DNA synthesizer. Overlapping oligonucleotides of 60-80 nucleotides from the positive and negative stands which are complementary among the adjacent oligonucleotides at their 3' ends can be synthesized with one of the commercial DNA synthesizers, such as one from Applied Biosystems, Inc. The oligonucleotides provide both the templates and primers (mutually primed synthesis) to generate the desired sequence in one-single step. After elongation is performed with T7 DNA polymerase, the segments are linked by a ligase. The oligonucleotides at the two ends of the genes are properly designed to include restriction enzyme sites, so that the synthesized genes can be inserted into the proper expression vector. The reagents to be prepared and the stepwise procedure is

described by Moore, D.D., *Current Protocols in Molecular Biology,* Supp. 6 § 8.2.8, Eds. Ausubel, F.M. et al, Wiley Intersciences (1990). This method is attractive because it easily allows construction of the large number of site-directed mutations needed to make the various mutant constructs, and because cytokine genes in general are relativity small, shorter than 600 nucleotides. All of the oligonucleotides, except the one with the specific mutation, may be shared for the individual constructs. Complete synthesized interferon genes have been made. *See* Edge, M.D. et al. *Interferon 7*, Ed. Gresser, I pp. 2-46 (Academic Press, London, 1986).

(v) Expression

The next step is to express the wild type and the mutated sets of cDNA in a eukaryotic or prokaryotic expression system and produce the native cytokine and the mutant cytokines, and then to purify the cytokines to produce sufficient amounts of each. For most cytokines which contain disulfide bonds or are glycosylated, the eukaryotic expression system is preferred because it assures disulfide bond formation and the proper folding of the polypeptide chains. Among the various eukaryotic expression systems, a preferred system for the expression of the foreign cytokine genes uses the expression vector derived from nuclear polyhedron protein gene of the insect virus baculovirus *(Autographa Californica)*. The virus can be grown in the insect cells. *Sprodoptera frugiperda* cells (sf9 cells). Luckow, V.A. and Summers, M.D., *Virology* 167:31 (1989). This expression system has been packaged into a convenient kit (designated "Max Bac Baculovirus Express System") and is sold by Invitrogen (San Diego, CA.).

For cytokines which do not contain a disulfide bond, such as IFN-γ, and for which a carbohydrate moiety either does not exist or is not involved in the binding to the cytokine receptor, the expression may be carried out in prokaryotic cells. When an *E. coli* expression system is used, the expressed cytokine proteins need to be solubilized, reduced to unfold the polypeptide chain, and allowed to renature to form the most favorable 3-dimensional structure. A preferred system is the FLAG Biosystem kit, offered by International Biotechnologies of Kodak (New Haven, CT.). This system also contains the reagents for the detection and purification of the non-fused protein. Monoclonal antibodies for most cytokines have been developed and are commercially available. These monoclonal antibodies can be used to affinity-purify the respective cytokines.

(vi) Conjugation

The next step is to conjugate the purified native and mutant cytokines with fatty acyl groups, preferably of about 8-14 carbons in length. An example of a preferred chemical reaction suitable to accomplish this conjugation using a 10-carbon fatty acyl group, which is adopted from the method of Carlsson et al. *Biochem. J.* 173:723 (1978), is as follows:.

In Reaction 1, 1-bromodecane (from Aldrich Chem. Co., Milwaukee, Wisconsin) is reacted with 2, 2'-dipyridyl disulfide (Aldrich) to form pyridyl decane disulfide. In Reaction 2, this product is reacted with AUC cytokine to form the decanoyl protein.

Before performing the conjugation reaction, however, the first step is to create a free SH group on the cytokine. The free SH group of the unpaired cysteine residue of a cytokine, which often is coupled to other sulfhydryl group-containing metabolites during biosynthesis, is first reduced by mild reducing conditions to free it from such metabolites. The mild reducing conditions, however, do not reduce the disulfide bonds buried inside the molecular backbone of the cytokine. After reduction, the reducing agent is removed by gel filtration or ion exchange chromatography. The treated cytokine is then reacted with the fatty acyl groups, which have been previously modified with an active group.

The native cytokine likely will not conjugate with the fatty acyl groups, as the native cytokine usually does not have any accessible, unpaired cysteine residues. However, for those native cytokines which do have accessible unpaired cysteine residues, they will also be conjugated to fatty acyl groups by the procedure described above. Thereafter, they can be analyzed for receptor binding/biological activity as described immediately below, to determine whether they are AUC cytokines. If this analysis reveals that they are not AUC cytokines, then the unpaired cysteine residue

may be replaced by a serine residue (to ensure that it does not conjugate with the fatty acyl group), and another residue at another location can be replaced with a cysteine residue. This substitution of a serine for a cysteine will not affect the receptor binding or biological activity. The conjugation reaction will only create fatty acylation of such cytokines at the one unpaired cysteine residue, and not elsewhere.

(vii) Receptor binding/biological activity

The last step is to analyze and compare the receptor-binding and biological activity of the native cytokine and the fatty acyl-conjugated mutant cytokine. Those mutant cytokine molecules with particular cysteine residue substitutions which have substantially the same receptor-binding and biological activity as the native cytokines are referred to as AUC cytokines.

2. Example: The preparation of AUC-IFN-α2

The steps described above in Section D.(i)-(vii) can be used, for example, to produce the AUC cytokine IFN-α2. IFN-α2 is a member of the family of interferon cytokines, and Table 1 below summarizes the pertinent key properties of this family, and more particularly includes information about the cysteine residues thereof.

There are 19 forms of IFN-α, one form of IFN-β, and one form of IFN-γ. All but two of the IFN-α have 4 cysteine residues, forming 2 disulfide bonds, between residue Nos. 1 and 99 (or 98 or 100) and between Nos. 29 and 39. Only IFN-α1 and IFN-αD have an unpaired cysteine residue (No. 86). IFN-β has 1 disulfide bond (between cysteine residue Nos. 31 and 141) and one unpaired cysteine residue (No. 17). IFN-γ has no cysteine residue.

TABLE 1. CYSTEINE RESIDUES OF HUMAN INTERFERONS

IFN	# Total Residues	# Cysteine Residues	S-S Bonds	Free Cysteine
IFN-α6	166	4	C1-C99, C29-C39	0
IFN-α5	166	4	C1-C99, C29-C39	0
IFN-α2	165	4	C1-C98, C29-C39	0
IFN-α1	166	5	C1-C99, C29-C39	C86
IFN-αD	166	5	C1-C99, C29-C39	C86
IFN-αH1	166	4	C1-C99, C29-C39	0
IFN-α8	166	4	C1-C99, C29-C39	0
IFN-αB	166	4	C1-C100, C29-C39	0
IFN-α4b	166	4	C1-C99, C29-C39	0
IFN-αC	166	4	C1-C99, C29-C39	0
IFN-αL	166	4	C1-C99, C29-C39	0
IFN-αJ1	166	4	C1-C99, C29-C39	0
IFN-αJ2	166	4	C1-C99, C29-C39	0
IFN-αI	166	4	C1-C99, C29-C39	0
IFN-αF	166	4	C1-C99, C29-C39	0
IFN-αWA	166	4	C1-C99, C29-C39	0
IFN-αGx-1	166	4	C1-C99, C29-C39	0
IFN-α76	166	4	C1-C99, C29-C39	0

IFN-α88	166	4	C1-C99, C29-C39	0
IFN-β	166	3	C31-C141	C17
IFN-γ	143	0	0	0

For preparing an AUC cytokine, the procedure described above in Section D.(i) to (vii) where an unpaired cysteine residue is introduced into the cytokine should be employed for most of the IFN, but not for IFN-α1, IFN-αD, and IFN-β. Because IFN-α1, IFN-αD, and IFN-β all have an unpaired cysteine residue, the procedure described in Section D.(vi) (where an unpaired cysteine residue is substituted by a serine and then a cysteine is substituted elsewhere) should instead be followed.

The fact that amino acid residue No. 86 is a serine or tyrosine residue in all IFN-α except IFN-α1 and IFN-αD suggests that the cysteine residue No. 86 in IFN-α1 and IFN-αD is not crucial. Thus, for IFN-α1 and IFN-αD, one would first try direct conjugation of a fatty acyl group to Cys 86 according to the techniques of Section D.(vi), and then use the methods of Section D.(vii) to determine if the conjugate remains receptor-binding and biological activities. If this substitution is not feasible or if the analysis reveals that the cytokine is not an AUC cytokine, one could replace Cys 86 with a serine residue and proceed to prepare an AUC-IFN-α1 and IFN-αD according to the methods of Section D.(vi).

Similar approaches can be used to prepare the AUC form of IFN-β. It is noted that if the native unpaired cysteine residue is replaced by a serine residue or another residue, and an unpaired cysteine residue is introduced replacing another residue in

a different location, the mutant cytokine will have two site-specific mutations, but only one site suited for conjugation with a lipophilic substance.

As Table 1 indicates, IFN-α2 has four cysteine residues forming two disulfide bonds. The nucleotide sequence of the cDNA gene and the deduced amino acid sequence are known and published. Using a hydrophicility analysis program provided by MicroGenie, which adopts the principles of Hopp, T.P. and Wood, K.R. *Mol. Immunol.* 20:483 (1983), a hydrophicility plot is made (not shown). The plot indicates regions or peptide segments of relatively high hydrophicility. Using the criteria discussed above in Section D., the amino acid residues selected for site-directed mutagenesis (*i.e.*, for substitution with cysteine residues) are: serine No. 11, arginine No. 22, lysine No. 31, glutamic acid No. 42, glutamic acid No. 51, serine No. 72, glutamic acid No. 113, serine No. 115, lysine No. 133, serine No.160, and Serine No. 163 (creating eleven mutant constructs in total).

The preferred method for preparing the native IFN-α2 gene and the 11 mutuant gene constructs is with the oligonucleotide synthesis method as discussed above in Section D.(iv). The preferred conjugation method is described in Section D.(vi).

The step-by-step procedure described above generally, and for IFN-α2 specifically, can be followed for preparing other AUC cytokines. As analyzed above, most native cytokines have even numbers of cysteine residues and do not contain an unpaired cysteine residue. For example, human EGF polypeptide is 53 amino acid residue in length containing 6 cysteine residues (3 disulfide bonds); human basic FGF polypeptide is 155 amino acid residue in length containing 4 cysteine residues (2

disulfide bonds), human IGF polypeptide is 70 amino acid residue in length containing 6 cysteine residues (3 disulfide bonds); human TNF-α polypeptide is 157 amino acid residue in length containing 2 cysteine residues (1 disulfide bond); and human β-NGF polypeptide is 188 amino acid residue in length containing 6 cysteine residues (3 disulfide bonds). Relatively few cytokines have odd numbers of cysteine residues and thus an unpaired cysteine residue. One is human IL-2 polypeptide, which is 153 amino acid residue in length containing 3 cysteine residues (1 disulfide bond). The unpaired cysteine residue No. 125 can be substituted to a non-cysteine residue without losing IL-2's receptor-binding and biological activity. E.P.A. application 136489. Thus, this cysteine residue (No. 125) provides a very likely site for conjugation.

E. Application of AUC Cytokines for Preparing Improved Reagents for Research and Diagnostic Assays

The present invention focuses mainly on the fatty acyl derivatives of AUC cytokines for local or mucosal therapeutic administration. However, these uses are not the only therapeutic applications of AUC cytokines. Cytokines, such as IL-2, IL-4, and EGF have been explored as binding molecules and vehicles for cytotoxins, such as pseudomonus exotoxin,to tumor cells expressing high densities of the receptors of the respective cytokines. The coupling of cytotoxins to the native cytokines by the conventional methods will yield heterogeneous conjugates. The coupling of cytotoxins to AUC cytokines via the unpaired cysteine residue will yield improved conjugates for the targeting of cytotoxins to cells.

Therapeutic applications are not the only applications of AUC cytokines. Several applications of AUC cytokines for providing useful reagents for research and

diagnosis may also be developed. In studying cytokine function, it is important to quantitate the levels of the cytokine in culture or in body fluids, the expression levels of the cytokine receptors on target cells, and/or the levels of receptor-expressing cells in a tissue. In certain diseases or conditions, the levels of these biological parameters may change. Monitoring these levels, therefore, can offer a means to determine or monitor disease status.

For such quantitative assays, which include immunochemical and cell-binding assays, cytokines conjugated with indicator substances (such as antibodies, horseradish peroxidase, alkaline phosphatase, fluorescein substances, or biotin, for use in conjunction with avidin-coupled indicators) are useful reagents. In the conventional methods for preparing these conjugates, the coupling is made through the ϵ-amino groups of the lysine residues. However, as elucidated above, the conjugates formed in this manner will be heterogeneous in terms of the numbers and the locations of the conjugated groups. Also, the activity and receptor binding properties of some of these conjugated cytokine molecules may be affected by the coupling of the modifier group to a lysine residue which is in or close to the binding site. Conjugation of these modifier groups via the single unpaired cysteine residue of an AUC cytokine, using the methods described above, can minimize or eliminate these problems and thus provide improved reagents for research and diagnosis. Cytokines are better than receptor-specific antibodies for studying the receptors, because cytokines usually have higher affinities than antibodies for their respective receptors. Cytokines are smaller in size than antibodies and thus are especially suitable for staining tissue sections,

where tissue penetration and diffusion of the reagents is important.

It should be understood that the terms, expressions and examples herein are exemplary only and not limiting, and those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. All such equivalents are intended to be encompassed by the following claims.

What Is Claimed Is:

1. A cytokine which is site-specifically modified to include one unpaired cysteine residue located away from the receptor-binding site so that conjugation of a lipophilic substance to the unpaired cysteine residue does not significantly affect the receptor binding or biological activity of the conjugated product.

2. A cytokine of claim 1, which is one of the following: α-interferon other than interferon-α1 and interferon-αD, γ-interferon, interleukin-1, interleukin-2, tumor necrosis factor, epidermal growth factor, fibroblast growth factor, insulin-like growth factor, platelet-derived growth factor, nerve growth factor, and ciliary nerve trophic factor.

3. A cytokine of claim 1, in which the free SH group is conjugated with a property-modifying group.

4. A conjugated cytokine of claim 3, in which the property-modifying group is selected from the group consisting of an antibody, biotin, fluorescein chromophore, horeseradish peroxidase, and a lipophilic substance.

5. A conjugated cytokine of claim 3, in which the property-modifying group is a fatty acyl group or a lipophilic and uncharged peptide.

6. The conjugated cytokine of claim 5 wherein the fatty acyl group is 6, 8, 10, 12, 14, 16, or 18 carbons in length.

7. The conjugated cytokine of claim 6 wherein the fatty acyl group is 8-14 carbon, saturated, and unbranched.

8. The conjugated cytokine of claim 6 wherein the conjugated cytokine is

IFN-α2 which has a cysteine residue replacing serine No. 11, arginine No. 22, lysine No. 31, glutamic acid No. 42, glutamic acid No. 51, serine No. 72, glutamic acid No. 113, serine No. 115, lysine No. 133, serine No. 160, or serine No. 163, and a fatty acyl group linked to the substituted cysteine residue.

9. A pharmaceutical preparation comprising a derivatized cytokine of claim 5 suitable for administration to a patient for treating a localized disease or condition.

10. A cytokine having one unpaired cysteine residue located away from the receptor-binding site conjugated to a lipophilic substance, and whereby such conjugation does not significantly affect the receptor binding or biological activity of the conjugated product.

11. The cytokine of claim 10 which is one of the following: interferon-α1, interferon-αD, or interferon-β.

12. A method of making a lipophilized cytokine comprising:

substituting an unpaired cysteine residue on a cytokine molecule at a site away from the cytokine's receptor-binding site so that conjugation of a lipophilic substance to the unpaired cysteine residue does not significantly affect the receptor binding or biological activity of the conjugated product; conjugating a lipophilic substance to the unpaired cysteine residue.

13. A pharmaceutical composition comprising a derivatized cytokine of claim 10

suitable for administration to a patient for treating a localized disease or condition.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/10889

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 13/00; A61K 45/05
US CL :530/351; 424/85.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/351; 424/85.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Vol. 47, issued 05 December 1986, I. Pastan et al., "Immunotoxins", pages 641-648, see entire document.	1-13
Y	J. Immunol., Vol. 147, No. 7, issued 01 October 1991, O. Bakouche et al., "Acylation of cell-associated IL-1 by palmitic acid", pages 2164-2169, see entire document.	1-13
Y	Cancer Res., Vol. 51, No. 13, issued 01 July 1991, T. Utsumi et al., "Preparation and characterization of liposomal-lipophilic tumor necrosis factor", pages 3362-3366, see entire document.	1-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
09 March 1993

Date of mailing of the international search report
15 MAR 1993

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Form PCT/ISA/210 (second sheet)(July 1992)★